Pharmacological chaperones for human α -N-acetylgalactosaminidase

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Schindler/Kanzaki disease is an inherited metabolic disease with no current treatment options. This neurologic disease results from a defect in the lysosomal α -N-acetylgalactosaminidase (α -NAGAL) enzyme. In this report, we show evidence that the iminosugar DGJNac can inhibit, stabilize, and chaperone human α -NAGAL both in vitro and in vivo. We demonstrate that a related iminosugar DGJ (currently in phase III clinical trials for another metabolic disorder, Fabry disease) can also chaperone human α -NAGAL in Schindler/Kanzaki disease. The 1.4- and 1.5-Å crystal structures of human α -NAGAL complexes reveal the different binding modes of iminosugars compared with glycosides. We show how differences in two functional groups result in >9 kcal/mol of additional binding energy and explain the molecular interactions responsible for the unexpectedly high affinity of the pharmacological chaperones. These results open two avenues for treatment of Schindler/Kanzaki disease and elucidate the atomic basis for pharmacological chaperoning in the entire family of lysosomal storage diseases.

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The lysosome is an acidic organelle containing dozens of enzymes that catalyze the breakdown of cellular metabolites. One lysosomal enzyme is α -N-acetylgalactosaminidase (α -NAGAL) (E.C. 3.2.1.49), a hydrolase that catalyzes removal of terminal α -linked N-acetylgalactosamine (α -GalNAc) and (less efficiently) galactose monosaccharides (1). Substrates for human α -NAGAL include glycans found in O-linked glycosylation, blood group A antigens, and others with α -GalNAc glycosides. In humans, mutations in the *NAGA* gene that codes for α -NAGAL lead to a loss of enzymatic activity in the lysosome and the subsequent accumulation of substrates, which eventually results in the autosomal recessive Schindler/Kanzaki disease (2–4). Schindler/Kanzaki disease belongs to the lysosomal storage disease family, which includes such diverse diseases as Tay-Sachs, Sandhoff, Gaucher, and Fabry diseases. Lysosomal storage diseases are characterized by the accumulation of substrates in the absence of a functional enzyme, leading to progressive deterioration in patients. Schindler/Kanzaki disease presents a wide range of symptoms in patients but is primarily characterized by neuronal pathologies (5–11). In humans, the *NAGA* gene is most closely related to the *GLA* gene, which codes for α -galactosidase (α -GAL A), and mutations in *GLA* lead to Fabry disease (12–14).

There are three principal treatment strategies currently approved or in clinical trials for lysosomal storage diseases. The first is enzyme replacement therapy, where the missing enzymatic activity is provided by regular injections of enzyme purified from recombinant sources. This therapy has been approved for the treatment of Fabry, Gaucher, and Pompe diseases and mucopolysaccharidosis-I, -II, and -VI (15). A second strategy is substrate reduction therapy, where an inhibitor of an enzyme upstream in a biosynthetic pathway leads to reduced substrate accumulation (16). A third approach is pharmacological chaperone therapy, where the mutant enzyme is stabilized by the addition of a small-molecule chaperone. This strategy has been proposed for Gaucher and

Pompe diseases and is currently in phase III clinical trials for Fabry disease (17, 18). Approximately 50% of Fabry disease mutations lead to defects in the folding or stability of the enzyme, and this subset responds to pharmacological chaperone in cellular studies (18). Additionally, the pharmacological chaperone strategy allows the possibility of treatment of lysosomal storage diseases with neurological manifestations, because small-molecule chaperones can potentially cross the blood–brain barrier, whereas macromolecular enzymes are typically unable to cross into the brain. To prevent the occurrence of lysosomal storage diseases, a modest 5–15% threshold of enzymatic activity may be sufficient (17, 19–21).

The most promising pharmacological chaperone in the clinic is the iminosugar analog of galactose, 1-deoxygalactonojirimycin (DGJ), used for the treatment of Fabry disease (22). DGJ can bind and stabilize α -GAL A at neutral pH in the endoplasmic reticulum (ER), allowing it to traffic to the lysosome, where it then dissociates at low pH (18, 22–28). Paradoxically, the addition of a small-molecule competitive inhibitor of an enzyme leads to an increase in the amount of activity of the enzyme, which slows the progression of the disease.

In this report, we sought specific pharmacological chaperones for the human α -NAGAL enzyme. We found that DGJ can bind, inhibit, and chaperone α -NAGAL nearly as well as it chaperones α -GAL A. Because DGJ has promiscuity for a number of lysosomal enzymes, we tested a related molecule with improved specificity for α -NAGAL. We synthesized an iminosugar, 2-acetamido-1,2-dideoxy-D-galactonojirimycin (DGJNac), specific for α -NAGAL (29, 30), and we examined its ability to bind, inhibit, and chaperone human α -NAGAL. We performed enzyme kinetics experiments to measure the binding and inhibitory properties of the two compounds on human α -NAGAL. We show that DGJNac and DGJ are able to protect human α -NAGAL from proteolytic degradation. We determined high-resolution crystal structures of the complexes of DGJNac and DGJ bound to human α -NAGAL at 1.4 and 1.5 Å, respectively, and a glucose-soaked structure with an empty active site at 1.6 Å. We then tested the ability of DGJNac and DGJ to chaperone α -NAGAL in cellular assays. Overall, our experiments reveal the atomic basis for the tight binding of iminosugars to lysosomal glycosidases. We show that both DGJNac and DGJ are suitable pharmacological chaperones for α -NAGAL, and the potency of DGJNac in vitro is ~10-fold greater. We propose DGJNac and DGJ as potential pharmacological chaperones for Schindler/Kanzaki disease patients.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 4DO4, 4DO5, and 4DO6).

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Results

Inhibition of α -NAGAL by the Iminosugars DGJNAc and DGJ. To test whether DGJNAc and DGJ would make suitable pharmacological chaperones for human α -NAGAL, we first measured their binding in enzymatic inhibition assays. As predicted from their similarity to the catalytic products of the α -NAGAL reaction, both DGJNAc and DGJ are inhibitors of α -NAGAL. DGJNAc is a tight binding inhibitor of α -NAGAL, with a K_i of 51 nM, whereas DGJ binds 29-fold weaker with a K_i of 1.5 μ M (Fig. 1). We also tested the ability of catalytic products to inhibit human α -NAGAL: GalNAc binds 250,000-fold weaker with a K_i of 12.7 mM (Fig. 1), and galactose is a very weak inhibitor, where concentrations up to the assay limit of 100 mM did not inhibit the enzyme. By comparison, in the interaction exploited in clinical trials of pharmacological chaperone therapy for Fabry disease, DGJ has a K_i of 40 nM for α -GAL A. Because the affinities of DGJNAc for α -NAGAL and DGJ for α -GAL A are similar, DGJNAc has potency in the appropriate range for pharmacological chaperone therapy in Schindler/Kanzaki disease.

Fig. 1. Enzymatic inhibition of α -NAGAL by DGJ, DGJNac, and GalNac. (A) DGJNac (red), DGJ (yellow), and GalNac (blue) were tested for their ability to inhibit the α -NAGAL-catalyzed cleavage of pNP- α -GalNac. (B) The measured IC_{50} values were converted into K_i values as described in the text. K_M values for substrates are also shown. (C) Three enzymes with related activities (α -NAGAL, α -GAL A, and GLB1) were tested in enzymatic assays in the presence of no inhibitor (Left), 20 μ M DGJNac (Center), or 20 μ M DGJ (Right). DGJNac shows increased specificity over DGJ and inhibits only α -NAGAL.

Fig. 2. DGJ and DGJNAc confer protection against protease digestion of α -NAGAL. (A and B) Wild-type (wt) α -NAGAL was preincubated with 0–100 μ M DGJNAc (A) or DGJ (B) before thermolysin digestion and SDS/PAGE. The intensity of the undigested α -NAGAL band indicates compound-induced protection from proteolysis. (C and D) Densitometry plots of the wild-type (C) and E367K mutant (D) α -NAGAL bands at different compound concentrations show the higher potency of DGJNAc over DGJ.

To examine the specificity of DGJNAc and DGJ, we performed the inhibition assays with two lysosomal enzymes with closely related activities, α -GAL A and GLB1 (also known as β -galactosidase). DGJNAc is highly specific for α -NAGAL, whereas DGJ inhibits α -GAL A better than α -NAGAL and partially inhibits GLB1 (Fig. 1C), in agreement with previous enzymatic and crystallographic studies (18, 25, 27, 31–34).

Protease Protection Conferred by Iminosugar Binding. We tested whether DGJNac and DGJ could limit the proteolytic degradation of α -NAGAL when exposed to limited thermolysin digestion (35). Native α -NAGAL protein is resistant to many proteases, including trypsin, thermolysin, and pepsin, but addition of denaturant allowed for digestion. α -NAGAL was preincubated with different concentrations of DGJNac or DGJ, and then equilibrated in 4 M urea before limited digestion and SDS/PAGE, revealing the amount of undigested α -NAGAL. The digestions show that, at pH 7.2 (mimicking the pH in the ER), both DGJNac and DGJ are effective pharmacological chaperones for human α -NAGAL, where both compounds protect α -NAGAL from proteolysis, with DGJNac protecting at ~ 10 -fold lower concentrations (Fig. 2). Both wild-type and the Schindler/Kanzaki mutant E367K α -NAGAL behave similarly in the digestion assay.

Atomic Basis of Iminosugar Interaction with Glycoside Hydrolase Family 27 Enzymes. To investigate the atomic basis of DGJNac and DGJ binding to α -NAGAL, we determined three crystal structures of α -NAGAL soaked with DGJNac, DGJ, or glucose (Fig. 3 and Table S1). Comparison of the new structures with the previously determined complexes of α -NAGAL with GalNac and Gal (36) reveals a highly favorable ionic interaction in the

Fig. 3. Pharmacological chaperone binding in the active site of human α -NAGAL. (A–C) σ_A -weighted 2Fo-Fc electron density maps of α -NAGAL soaked with DGJNac, DGJ, and glucose respectively. A and B are contoured at 1.8σ around the ligand density and C at 1.5σ . (D) Schematic indicating the key interaction between Asp156 and DGJNac or GalNac. (E and F) Superposition of structures of iminosugar complexes comparing DGJNac (red) and DGJ (yellow) with their sugar analogs GalNac (blue) and Gal (green), and the location of the catalytic nucleophile Asp156. (G) Plots of the distances of Asp156 to the ligand C1 and heteroatom, indicating the different mode of binding of the iminosugars and sugars. The colors are as in E and F, the dashed line shows the mean of four grouped measurements, and brackets show *P* values from Student *t* tests of the paired data.

iminosugar complexes (Fig. 3D). In sugar complexes from this family of enzymes, the catalytic nucleophile is poised to attack C1 of the ligand (31, 33, 36–40). The distances from the nucleophile D156 to C1 of GalNac (2.97 Å) and to C1 of Gal (3.06 Å) are shorter in the sugar complexes, where the *syn* lone pair of D156 points directly at C1 of the sugar, in position for nucleophilic attack. However, when an iminosugar is bound in the α -NAGAL active site, D156 shifts away from C1 (3.21 and 3.28 Å for DGJNac and DGJ, respectively) and toward N5 of the ligand (Fig. 3E–G). The DGJNac and DGJ iminosugars bind more like each other than like the GalNac and Gal sugars. When iminosugars are compared with sugars, the distances between D156 and the ligand shift by statistically significant amounts, with $P < 0.01$.

DGJNac and DGJ Chaperoning of Human α -NAGAL in Cells. To examine the ability of DGJNac and DGJ to chaperone wild-type α -NAGAL *in vivo*, we transfected human embryonic kidney (HEK 293T) cells with plasmids encoding FLAG-tagged human α -NAGAL in the presence or absence of the compounds. Immunoblots with anti-FLAG antibodies revealed higher levels of α -NAGAL protein expressed in cells cultured in the presence of DGJNac or DGJ (Fig. 4A and B). Despite the higher potency of DGJNac *in vitro*, cells cultured with DGJNac showed a lesser increase in α -NAGAL protein expression, possibly due

to differential uptake of DGJ and DGJNac in cells (30). Both DGJ and DGJNac showed statistically significant increases in protein levels compared with no compound (100 μ M DGJNac and 500 μ M DGJ have $P < 0.05$ in paired Student *t* tests). The ability of DGJ to chaperone wild type α -GAL A in cellular assays led to its eventual development as a clinical candidate (22).

To explore the effect of the compounds on mutant forms of α -NAGAL found in Schindler/Kanzaki disease patients, we performed subcellular fractionation assays to separate lysosomal and ER-resident forms of α -NAGAL (Fig. 4C). Overexpressed α -NAGAL shows two distinct bands when blotted with a polyclonal antibody. Subcellular fractionation revealed that the higher molecular weight band (Fig. 4C, white arrowhead) fractionates with the ER marker Calnexin, retains the C-terminal FLAG tag, and has no Endo H-resistant glycans, indicative of an immature ER protein. The lower molecular weight band (Fig. 4C, black arrowhead) fractionates with the lysosomal marker LAMP2, loses the C-terminal FLAG tag, and has Endo H-resistant glycans, indicative of a mature lysosomal protein.

DGJNac and DGJ can restore wild-type cellular distribution to the S160C α -NAGAL, a Schindler/Kanzaki disease variant form. In the absence of pharmacological chaperone, cells expressing the S160C α -NAGAL mutant show little mature α -NAGAL (and almost no immature α -NAGAL, presumably due to ER-associated

Fig. 5. Thermodynamic cycle for binding of ligands to α -NAGAL. A $\Delta\Delta G$ representation of ligand binding to human α -NAGAL shows that the presence of an *N*-acetyl group of GalNAc and DGJNAc provides ~ 2 kcal/mol of binding energy, whereas the ring nitrogen provides >7 kcal/mol. At the corners of the cycle, DGJNAc binds to α -NAGAL $>10^6$ -fold tighter than galactose does.

Replacement of the ring oxygen in hexose sugars with nitrogen in iminosugars provides >7 kcal/mol of additional binding energy. The two substitutions taken together lead to >9 kcal/mol improved binding, or better than 10^6 -fold improvement in binding affinity, a remarkable difference for highly similar compounds.

Because of the similarity of pharmacological chaperones to natural ligands, they can have off-target binding. For example, 20 μ M DGJ (a clinically relevant concentration for targeting α -GAL A in Fabry disease treatment) inhibits α -NAGAL by 70% and GLB1 by 25% in vitro (Fig. 1). In cellular assays, 10 μ M DGJ inhibits both α -NAGAL by 63% and GLB1 by 25% (18), potentially affecting the degradation of substrates with terminal α -GalNAc and β -Gal glycosides. Thus, the potency and efficacy of DGJ for α -NAGAL are both encouraging and problematic pharmacologically. In Schindler/Kanzaki disease, DGJ may be useful as a pharmacological chaperone. However, the DGJ- α -NAGAL interaction also represents unwanted off-target binding of the DGJ- α -GAL A interaction central to pharmacological chaperoning for Fabry disease. The 1.6 μ M K_i of DGJ for α -NAGAL is well below the therapeutic target concentration for treatment of Fabry disease, so off-target binding to α -NAGAL is expected. In contrast, DGJNAc contains an *N*-acetyl group that sterically precludes it from binding to α -GAL A and GLB1 (31–34), so it has improved selectivity for α -NAGAL.

In contrast to traditional enzyme inhibitors used in the clinic, effective pharmacological chaperones must meet additional criteria (17). Traditional inhibitors simply bind the active site of their target with high potency, rendering the enzyme ineffective due to competitive inhibition. On the other hand, pharmacological chaperones must not only bind to the target enzyme with high potency, but they must also dissociate from the active site of the enzyme for catalysis to occur. Additionally, an effective pharmacological chaperone must also distribute efficiently to the ER, where folding of the target enzyme occurs.

DGJNAc, the iminosugar analog of GalNAc, has been proposed as a possible pharmacological chaperone for human α -NAGAL. DGJNAc was originally hypothesized to be an inhibitor of α -NAGAL by extension of observations on the Fabry disease equivalents (DGJ and α -GAL A) (17). However, a report of the specific synthesis and testing of the compound appeared only

recently (29) [and not in a sometimes-cited earlier paper (42)]. Here, we describe biochemical, structural, and cellular studies on the interaction and chaperoning of human α -NAGAL by DGJNAc.

Our experiments point to DGJNAc and DGJ as reasonable candidates for pharmacological chaperones for Schindler/Kanzaki disease, as both compounds meet the affinity criterion suggested for developing new pharmacological chaperones. [A threshold of IC_{50} of <10 μ M is recommended for promising pharmacological chaperone lead compounds (17).] Our protease protection studies show that both DGJNAc and DGJ are capable of reducing the amount of α -NAGAL digested by protease, indicating that they shift the folding equilibrium toward the folded state. In cellular assays, we show that DGJNAc and DGJ are able to increase the amount of wild-type α -NAGAL protein produced by mammalian cells. Additionally, we show that DGJNAc and DGJ are able to increase the amount of S160C α -NAGAL (a Schindler/Kanzaki disease variant form) delivered to the lysosome compared with controls. DGJNAc and DGJ increase cellular expression of α -NAGAL by 20–30%. This increase is comparable to the chaperoning seen with the compounds DGJ and isofagomine, which have been tested in clinical trials for Fabry and Gaucher diseases (18, 43).

Because the population of patients with Schindler/Kanzaki disease is extremely small, it is unlikely to be an active target for clinical development by the pharmaceutical industry, and the neurological manifestations of the disease make enzyme replacement therapy unsuitable. We suggest that DGJ (which has already succeeded in phase I clinical trials, demonstrating its safety in humans) might be repurposed for use as a pharmacological chaperone in the Schindler/Kanzaki disease patient population. Alternatively, DGJNAc (which has higher potency than DGJ for α -NAGAL in vitro but unexpectedly shows less efficacy in cellular experiments) might be adapted into a more effective pharmacological chaperone used in compassionate care for Schindler/Kanzaki disease. For example, iminosugar compounds for Gaucher disease were developed into clinical candidates by derivatization of the heterocyclic nitrogen with alkyl substituents (44, 45).

The role of α -NAGAL in cancer is controversial (46). It is well established that α -NAGAL in the serum is a biomarker for cancers including melanoma and that α -NAGAL activity is raised in patients with large tumor burdens (47). However, others have proposed a causal link between α -NAGAL and cancer progression: α -NAGAL in the serum is hypothesized to deglycosylate vitamin D binding protein, removing the latter's ability to activate macrophages, leading to immunosuppression (48). The DGJNAc molecule we describe here, a specific inhibitor of α -NAGAL, allows for direct testing of this hypothesis. If the proposed mechanism of α -NAGAL exacerbating cancer progression proves to be correct, DGJNAc may then be useful in the treatment of cancer by reducing immunosuppression in patients.

In summary, the structures, results, and design principles reported here will improve therapeutic approaches toward protein folding diseases. We have synthesized and tested DGJNAc, a compound for the treatment of Schindler/Kanzaki disease, showing that it binds and chaperones human α -NAGAL both in vitro and in vivo. We have established that DGJ, a compound currently in clinical trials for Fabry disease, could be repurposed to treat Schindler/Kanzaki patients, who currently have no treatment options available to them. By characterizing the inhibitory properties of DGJNAc toward α -NAGAL, we describe a compound that can be used to directly test the role of α -NAGAL in immunosuppression of several cancers. If the role of α -NAGAL in immunosuppression is proven, DGJNAc, as a high-affinity inhibitor of α -NAGAL, might be useful as an anticancer compound.

Materials and Methods

For biochemistry and crystallography experiments, α -NAGAL was produced in Tn5 insect cells. Crystal structures of N201Q α -NAGAL, an engineered variant

that produces high-quality crystals (36), were solved with DGJ bound, DGJNac bound, or with an empty active site. DGJNac was synthesized as described (29). For limited proteolysis studies, purified wild-type or E367K α -NAGAL was incubated overnight in 4 M urea, pH 7.2, in the presence and absence of DGJ or DGJNac. Samples were digested briefly with thermolysin and analyzed by SDS/PAGE. Cellular studies used HEK 293T cells transfected with FLAG-tagged wild-type, S160C, or R329W α -NAGAL. Density gradient cellular fractionation was used to analyze the cellular distribution of wild-type and mutant forms of α -NAGAL, and the ability of DGJ and DGJNac to rescue mutant forms of α -NAGAL. For complete methods, see *SI Materials and Methods*.

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